ELSEVIER

Vaccine 20 (2002) 2278–2286

Vaccine

www.elsevier.com/locate/vaccine

Immunisation of dairy cattle with recombinant *Streptococcus uberis* GapC or a chimeric CAMP antigen confers protection against heterologous bacterial challenge

Michael C. Fontaine [a], Jose Pérez-Casal [a], Xin-Ming Song [a], Jim Shelford [b], Philip J. Willson [a], Andrew A. Potter [a,*]

[a] *Veterinary Infectious Disease Organisation, University of Saskatchewan, 120 Veterinary Road, Saskatoon, Sask., Canada S7N 5E3*
[b] *Department of Animal Science, University of British Columbia, Vancouver, British Columbia, Canada*

Received 26 September 2001; received in revised form 8 January 2002; accepted 31 January 2002

Abstract

The *gapC* genes, encoding the cell surface-associated GapC proteins of *S. uberis* and *S. agalactiae*, have been cloned and sequenced. To identify potential vaccine candidates against *S. uberis*-induced bovine mastitis, lactating dairy cows were vaccinated with either (6 × His)GapC of *S. uberis* or *S. dysgalactiae*, or with a chimeric CAMP-factor antigen, CAMP-3. For 7 days following heterologous challenge with *S. uberis*, milk somatic cell counts were determined to assess differences in the severity of mastitis between vaccinates and an unvaccinated control group. Vaccination with *S. uberis* (6 × His)GapC or CAMP-3 resulted in a significant reduction in inflammation on several days post-challenge, most significantly for the former antigen. Inflammation was not reduced in *S. dysgalactiae* (6 × His)GapC vaccinates, suggesting that it does not confer cross-species protection. © 2002 Elsevier Science Ltd. All rights reserved.

Keywords: *Streptococcus uberis*; Glyceraldehyde-3-phosphate dehydrogenase; CAMP-factor; GapC; Mastitis; Vaccine

1. Introduction

Bovine mastitis, an inflammation of the mammary gland, is the single most important factor contributing to economic losses to the dairy industry [1]. Several streptococcal species are capable of causing infections that result in mastitis, including *Streptococcus uberis*, *S. agalactiae* and *S. dysgalactiae*. Among these species, *S. uberis* is particularly problematic due to the fact that this so-called 'environmental streptococcus' is ubiquitous in the dairy environment [2–4] and is predominantly associated with sub-clinical mastitis cases, resulting in reduced, and poor quality milk yields. The development of vaccines against mastitis-causing pathogens has been slow, partly due to the prerequisite that infection must be controlled without induction of a significant inflammatory response, since this in itself contributes to the disease condition [5]. Currently, prophylactic practices including antibiotic therapy and teat disinfection are relied upon to minimise the spread of infection. However, these measures are often inadequate, simply because animals are constantly being re-exposed to infection from their surrounding environment. Therefore, there is a clear requirement for an effective vaccine to augment conventional methods of preventing infection.

The factors contributing to the pathogenesis of *S. uberis* disease are not well understood, although several potential virulence determinants have been described. Resistance to phagocytosis is conferred by a hyaluronic acid capsule [6], and the organism has also been shown to adhere to and actively invade bovine epithelial cells via a receptor-mediated endocytosis mechanism, where it is able to persist without harming host cells [7]. *S. uberis* also produces hyaluronidase [8], and a plasminogen activating protein, PauA [9,10], which may aid in the dissemination of the bacterium into tissues surrounding the site of infection. Other potential virulence determinants include neutrophil toxin [7], lactoferrin binding proteins [11], and CAMP-factor [12,13]. Although a specific role for CAMP has yet to be determined, it causes the lysis of erythrocytes pre-treated with staphylococcal β-toxin, and due to immunoglobulin Fc-binding properties [14], an involvement in resistance to phagocytosis has also been suggested. The CAMP protein of *S. uberis* displays more than 60% homology with that of *S. agalactiae*, and

* Corresponding author. Tel.: +1-306-966-7465; fax: +1-306-966-7478.
E-mail addresses: Fontaine@sask.usask.ca (M.C. Fontaine), potter@sask.usask.ca (A.A. Potter).

PII: S0264-410X(02)00114-7

CAMP-specific antiserum are cross-reactive between the two species.

The plasma receptor (Plr) protein [15], or surface dehydrogenase (SDH) protein [16], a phosphorylating glyceraldehyde-3-phosphate dehydrogenase, has been implicated as a potential *S. pyogenes* virulence factor, which, in addition to plasma binding, also displays ADP-ribosylating activity [17]. This attribute potentially affects host cell gene transcription, enhancing disease pathogenesis [17]. Previous work in our laboratory, to be published elsewhere, revealed that GapC of *S. dysgalactiae*, a cell surface-associated Plr homologue, conferred significant protection against *S. dysgalactiae* infection in dry cows when used as a vaccine. Therefore, the potential for using GapC as a vaccine antigen against mastitis caused by other streptococci is apparent.

Reports of vaccination of dairy cows against *S. uberis* infection are limited. Sub-cutaneous vaccination with live *S. uberis*, and a soluble surface extract derived from the same bacteria, conferred some protection against experimental challenge [18]. However, although vaccination with live cells did result in protection against infection, this protection was less effective against heterologous strains [19]. Repeated vaccination with killed *S. uberis* cells also resulted in some degree of protection against subsequent challenge with the same strain [20], although vaccination did not reduce inflammation of the mammary gland. The most significant results to date derived from sub-cutaneous vaccination with a concentrated *S. uberis* culture supernatant, containing PauA, which resulted in between 37.5 and 62.5% protection from clinical disease following challenge with a heterologous strain [21].

To develop common vaccines against mastitis-causing streptococci, we have cloned and sequenced the *gapC* genes of *S. uberis* and *S. agalactiae*. Furthermore, a chimeric CAMP (CAMP-3) protein, comprising epitopes from the *S. uberis* and *S. agalactiae* CAMP-factors, has been constructed, to create a cross-reactive vaccine antigen which is more immunogenic than either CAMP-factor alone. Protection of lactating cattle against heterologous *S. uberis* challenge has been assessed following vaccination with CAMP-3, or the (6 × His)GapC protein of either *S. uberis* or *S. dysgalactiae*.

2. Materials and methods

2.1. Media and culture conditions

Escherichia coli strains were routinely cultured at 37 °C in Luria Bertani medium (LB; Difco), containing ampicillin (100 μg/ml) or carbenicillin (50 μg/ml). Streptococcal strains were cultured at 37 °C (5% CO_2) in Todd-Hewitt broth (Difco), supplemented with 0.5% (w/v) yeast extract (THBYE), or on Tryptic Soy Agar (TSA) plates containing 5% (v/v) sheep blood (PML microbiologicals, Mississauga, Ontario, Canada). Bacteriological analysis of milk samples was performed at the Animal Health Centre (Abbotsford, British Columbia). Aliquots of 100 μl were spread onto TSA-blood plates, and incubated as above for up to 48 h.

2.2. Molecular biological techniques

Restriction endonucleases and other modifying enzymes were supplied by Amersham Pharmacia and used according to the manufacturer's instructions. DNA fragments were purified using the GeneClean Spin Kit (BIO 101). Plasmid minipreps were performed with the QIAquick Spin Miniprep Kit (QIAGEN), and genomic DNA was extracted from streptococci using the NucleoSpin Tissue Kit (Clontech). PCR amplified DNA was cloned into pPCR-Script using the pPCR-Script Cloning Kit (Stratagene). PCR and sequencing primers are described in Table 1. Automated DNA sequencing was carried out at the CNRC sequencing facility (Plant Biotechnology Institute, University of Saskatchewan, Saskatchewan, Canada).

2.3. Cloning of streptococcal gapC genes

Design of the PCR primers *gapC*(*Nde*I) and *gapC*(*Bam*HI) for amplification of the *gapC* genes of *S. uberis* (ATCC 9927) and *S. agalactiae* (ATCC 27541) was facilitated by virtue of the high sequence similarity displayed at the 5′- and 3′-ends of the *gapC* genes of *S. dysgalactiae* and *S. equisimilis*, and the *plr* gene of *S. pyogenes*. PCR was carried out from genomic DNA of each strain, and the resulting ca. 1.0 kb fragments were cloned into pPCR-Script and used to transform *E. coli* XL10-Gold (Stratagene). Subsequently, the desired recombinant plasmids were identified and designated pMF501a and pMF501c, containing the PCR fragments from *S. uberis* and *S. agalactiae* respectively. Sequence analysis of both strands of the DNA inserts was performed using the T3, T7, *gapC*(fwd) and *gapC*(rev) primers. To allow expression of recombinant proteins, the inserts in pMF501a and pMF501c were liberated with *Nde*I and *Bam*HI, ligated to pET-15b, and used to transform *E. coli* DH5αF′*lacI*^{q}. The resulting plasmids were designated pMF521a and pMF521c, containing sequences from *S. uberis* and *S. agalactiae* respectively. For expression of (6 × His)GapC proteins, constructs were used to transform *E. coli* BL21(DE3).

2.4. Construction of the camp-3 gene

With a view to creating a cross-species-specific vaccine against *S. uberis* and *S. agalactiae*, a chimeric CAMP-factor-encoding gene was created, in which DNA sequences corresponding to amino acid (aa) residues 30–90 of *S. agalactiae* CAMP were cloned between those encoding the Leu_{90} and Lys_{91} residues of *S. uberis* CAMP. The published nucleotide sequences of the CAMP-encoding genes *cfb* [22] and *cfx* [13], from *S. agalactiae* and *S. uberis*

Table 1
PCR and sequencing primers

Primer	Sequence	Comments
gapC(*Nde*I)	5′-GCCGCCGCCATATGCTAGTTAAACTTGCTATTAACGG-3′	Forward primer for the amplification of *gapC*, annealing to nt 1–26. *Nde*I site underlined.
gapC(*Bam*HII)	5′-GCGGATCCTTATTTAGCGATTTTTGCAAAGTACTC-3′	Reverse primer for the amplification of *gapC*, annealing to nt 1011–985 (opposite strand). *Bam*HII site underlined.
gapC(fwd)	5′- ATCCACGCTTACACTGGTG-3′	Forward primer for sequencing of *gapC*, annealing at nt 532–550.
gapC(rev)	5′-CCAGTGTAAGCGTGGATAGTAGTC-3′	Reverse primer for sequencing of *gapC*, annealing at nt 548–525 (opposite strand).
T3	5′-ATAACCCTCACTAAAG3′	Universal primer for T3 promoter.
T7	5′-GTAAAACGACGGCCAGT-3′	Universal primer for T7 promoter.
CAMP-1	5′-AAAAAAGGATCCAATCAAATAAATGTTAGTCAACCA-3′	Forward primer, annealing to nt 91–112 of the *S. uberis* CAMP coding sequence. *Bam*HI site underlined.
CAMP-2	5′-AAAAACCATGGCTACTCGAGATTTTCAACAGCT GAATTGCTGAATTAAC-3′	Reverse primer, annealing to nt 267–238 (opposite strand) of the *S. uberis* CAMP coding sequence. *Xho*I, and *Nco*I underlined.
CAMP-3	5′-AAAAAACTCGAGCAAGTGACAACTCCACAAGTGG-3′	Forward primer, annealing to nt 91–102 of the *S. agalactiae* CAMP coding sequence. *Xho*I site underlined.
CAMP-4	5′-AAAAAACCATGGCTAAGGCCTTAATTTTT CCACGCTAGTAATAGCCTC-3′	Reverse primer, annealing to nt 261–235 (opposite strand) of the *S. agalactiae* CAMP coding sequence. *Stu*I, and *Nco*I sites underlined.
CAMP-5	5′-AAAAAAGCGCTAAAACTTCACTTAGAGCTAATCCTG-3′	Forward primer, annealing to nt. 271–295 of the *S. uberis* CAMP coding sequence. *Eco*47-3 site underlined.
CAMP-6	5′-AAAAACCATGGTCATTACTGTAGAGCAGTATTTA ATGCTTC-3′	Reverse primer, annealing to nt 777–751 (opposite strand) of the *S. uberis* CAMP coding sequence. *Nco*I site underlined.
His-CAMP-1	5′-AAAAAACATATGTCCAATCAAATAAATGTTAGTCAACC-3′	Forward primer for cloning into pET-15b. *Nde*I site underlined.
His-CAMP-2	5′-TTTTTGGATCCTTACTGTAGAGCAGTATTTAATGC-3′	Reverse primer for cloning into pET-15b. *Bam*HI site underlined.
556-1	5′-GTGTGGAATTGTGAGCGG-3′	Forward primer for sequencing of cloned inserts in pAA556a, annealing to nt 1791–1808.
556-2	5′-CTCCCTGCCTCTGTC-3′	Reverse primer for sequencing of cloned inserts in pAA556a, annealing to nt 1979–1965 (opposite strand).

respectively, were used to design PCR primers, allowing construction of the chimeric CAMP-encoding gene, *camp-3*. A PCR fragment encoding aa residues 30–90 of *cfx* was amplified with the primers CAMP-1 and CAMP-2. The fragment was cloned into the expression vector pAA556a (which contains OmpF N-terminal-encoding sequences, directing export of OmpF fusion proteins to the *E. coli* cell surface) using the primer encoded *Bam*HI and *Nde*I restriction sites, and the resulting construct was designated pPolyCAMP-1. A second PCR fragment, encoding residues 31–87 of *cfb*, was amplified with the primers CAMP-3 and CAMP-4 and cloned into pPoly-CAMP-1 using *Xho*I and *Nco*I sites to give pPolyCAMP-2. Finally, a third PCR fragment, encoding residues 91–258 of *cfx*, was amplified with the primers CAMP-5 and CAMP-6 and digested with *Eco*47-3 and *Nco*I. This fragment was cloned into *Stu*I/*Nco*I digested pPolyCAMP-2, and the resulting construct, pPolyCAMP-3, contained a chimeric gene, *camp-3*, encoding a protein of 317 aa, with a calculated M_r of 34,956 Da and a pI of 5.5. The *camp-3* construct was sequenced in both directions using the primers 556-1 and 556-2. To provide enough pure protein for subsequent vaccine studies, an *Nde*I/*Bam*HI fragment containing the *camp-3* region of pPolyCAMP-3, minus the OmpF signal sequence, was cloned into pET-15b to create the plasmid pET-CAMP-3, encoding a (6 × His)CAMP-3 fusion protein. DNA sequence analysis of this construct revealed that the CAMP-3 ORF was not fused to the peptide encoding the histidine tag, but that its expression was still controlled by IPTG (data not shown).

2.5. Nucleotide sequence accession numbers

The nucleotide sequences of the *S. uberis* and *S. agalactiae gapC* genes were submitted to GenBank under the accession numbers AF421899 and AF421900, respectively.

2.6. Purification of recombinant proteins

Recombinant (6 × His)GapC proteins were purified under native conditions by metal chelate affinity chromatography, using Ni-NTA agarose (QIAGEN) according to the manufacturer's specifications. Purified proteins were buffer exchanged into 0.1 M PBS (pH 7.2) with BIOMAX-30 K filters (Millipore), and purities were estimated to be >95% by densitometry of SDS–PA gels.

The CAMP-3 protein was purified by anion exchange chromatography of a filtered lysate of an IPTG-induced culture of BL21(DE3) containing pET-CAMP-3. Cells were collected by centrifugation at 6000 × *g* for 10 min at 4 °C, washed in 0.1 M PBS (pH 7.2), and disrupted by

sonication. The volume of the soluble fraction was adjusted to 650 ml with 20 mM Na_2HPO_4 (pH 7.5), and filtered through a 0.22 μm filter (Millipore). Q-sepharose fast flow anion exchange resin was packed into an XK26/20 column to a bed height of 13 cm (70 ml column volume). The column was equilibrated with buffer A (20 mM Na_2HPO_4, pH 7.5), and the protein solution was passed through at a rate of 7 ml/min. The column was washed with 7.4 column volumes (CV) of buffer A, and protein was eluted with a gradient buffer (0% buffer A to 50% buffer B [buffer A + 1 M NaCl, pH 7.5] over 12.85 CV and from 50 to 100% buffer B in 3.6 CV, and finally 100% of buffer B for 1.7 CV). The column eluate was monitored at 260 nm, and fractions were concentrated with BIOMAX-30 K filters. Analysis by SDS–PAGE and Western blot determined that the CAMP-3 protein eluted in the breakthrough and buffer A fractions. Densitometry of SDS–PA gels estimated protein purity to be >60%.

2.7. Immunisation and challenge of lactating cows

Immunisation and bacterial challenge of lactating Holstein cows was performed at the University of British Columbia Dairy Education and Research Centre (Agassiz, British Columbia). A total of 99 cows were screened for the presence of serum IgG against *S. uberis* whole cells, GapC, and CAMP. Four groups of 8 animals were selected for vaccination with a placebo, (6 × His)GapC of *S. uberis*, (6 × His)GapC of *S. dysgalactiae*, or CAMP-3. Each vaccine dose (2 ml) comprised 100 μg/ml of purified (6 × His)GapC, CAMP-3, or antigen-free placebo (0.85% (w/v) saline), and 30% VSA3 [23]. Cows received two subcutaneous injections in the neck at 36 (day 0) and 15 days prior to challenge. Eight days before challenge, milk samples from each quarter were analysed for the presence of bacteria, and infected animals were excluded from the trial. Subsequently, 6 cows from each group were challenged. Three hours before challenge, teats were washed with clean, warm water, dried, and alcohol swabbed. Milk samples were collected for somatic cell counts (SCC) and bacteriology. The left udder quarters remained unchallenged as controls. Three ml of inoculum was administered by intramammary infusion to the right quarters of each animal, containing 3.0×10^7 cfu/ml of an exponential-phase culture of *S. uberis* SU21 (clinical isolate obtained from Animal Health Laboratory, Alberta, Canada) suspended in 0.85% (w/v) saline. Milk samples were collected from all quarters, daily for 7 days post-challenge, for determination of SSC and bacteriology. All samples were stored on ice, and analysed within 48 h of collection. Clinical assessments of animals included measurement of rectal temperatures, and udder swelling (visual and palpated). A numerical score of 1 (normal) to 3.5 (severe mastitis) was assigned to each animal and used as a means of comparing the severity of mastitis among vaccine groups. Milk quality was assessed by the presence of clots.

2.8. Determination of GapC and CAMP-specific antibodies

Serum IgG titres were determined at the time of first and second vaccinations, at 8 days before challenge (day 28), and at 11 days post-challenge (day 47). Similarly, milk IgG titres were determined at days 21 and 43. Serum IgA titres were determined at days 21 and 47, and milk IgA titres were determined at days 21 and 43. Round-bottomed, 96-well microtitre plates (Nunc) were coated overnight at 4 °C with CAMP-3, and (6 × His)GapC of *S. uberis* and *S. dysgalactiae* (100 ng/well in 100 μl of carbonate buffer, pH 9.6), and blocked for 1 h at 37 °C with 200 μl of PBSTg. A total of 100 μl of test sample was added/well, and plates were incubated for 2 h at room temperature. After washing, alkaline phosphatase-conjugated goat anti-bovine IgG (H and L; Kirkegaard and Perry Labs, Inc.) was added (100 μl/well), and plates were incubated for 1 h at room temperature. Plates were washed, and alkaline phosphatase activity was detected at 405 nm following incubation with p-nitrophenyl phosphate in 1 M diethanolamine (pH 9.8) and 0.5 mM $MgCl_2$ for 1.5 h at room temperature.

Determination of milk IgG and IgA was carried out after treating milk with a commercially available rennin solution, as follows: one tablet of Rennet (CHR HANSEN) was dissolved in 40 ml of H_2O, and 0.1 ml of this solution was added to 2 ml of milk and incubated at room temperature for 4 h. Coagulated casein was pelleted by centrifugation at 3000 × *g* for 20 min, and the middle layer was removed (the top layer comprised fat) and analysed as for serum samples. Both serum and milk titres were determined by the intersection of the least-square regression of the OD_{405} versus logarithm of dilution with the OD_{405} obtained from wells containing no serum.

2.9. Somatic cell counts

Determination of SCC from milk samples was carried out at the Pacific Milk Analysis Laboratory (Chilliwak, British Columbia). Samples were collected in 14 ml polystyrene, round-bottomed tubes (Falcon) containing a preservative. SCC were fixed by mixing 0.5 ml of milk samples with 10 μl of fixative liquid (0.2 mg/ml cosine, 3.3% formaldehyde solution) for 18 h at 30 °C. Samples were diluted 1/100 in emulsifier electrolyte solution (12% ethanol, 0.02% Triton X-100, 0.1 M NaCl), and incubated at 80 °C for 10 min. After cooling to room temperature, SCC were determined with a Coulter counter.

2.10. Computational analyses

DNA and protein sequences were analysed using the Clone Manager 6 program (Scientific and Educational Software, Durham, North Carolina, USA) and compared using the Gap and PileUp applications of the GCG software package (Wisconsin Package Version 10.1; Genetics Computer

Group, Madison, Wisconsin, USA). Repeated measures analysis of variance of SCC among treatments, and over time, was performed using the SYSTAT 10 software package (SPSS Science, Chicago, USA).

3. Results

3.1. Analysis of cloned and expressed streptococcal gapC genes

We previously determined the presence of cell surface GapC in *S. dysgalactiae*, and the *gapC* gene has been cloned and sequenced (unpublished data). For the purposes of this study, we also confirmed the presence of cell surface-associated GAPDH activity on whole cells of the mastitis-causing pathogens *S. uberis* and *S. agalactiae* (data not shown).

Analysis of the PCR fragments cloned in pMF501a (*S. uberis*) and pMF501c (*S. agalactiae*) both revealed open reading frames (ORF) of 336 aa, encoding proteins with predicted relative masses of 35,906.43 Da and 35,933.39 Da, and pI's of 5.17 and 5.17, respectively. Analysis of purified, recombinant (6 × His)GapC proteins by SDS–PAGE indicated M_r's of ca. 40 kDa (data not shown), which closely matches the predicted ca. 38 kDa based on predicted fusion protein sequences. The predicted GapC sequence of *S. dysgalactiae* is highly similar to Plr, displaying 99.41% sequence identity, while the predicted aa sequences of the proteins described in this study display 92.26% (*S. uberis*) and 91.07% (*S. agalactiae*) identity with Plr. Based on the prior designation of the *S. dysgalactiae* gene as *gapC*, the *S. uberis* and *S. agalactiae* genes have been given the same designation.

3.2. Construction and purification of the CAMP-3 protein

The *cfb* and *cfx* genes of *S. agalactiae* and *S. uberis* share 66.67% similarity and 66.54% identity at the DNA level, and 69.29% similarity and 63.39% identity at the aa level. Alignment of the two protein sequences revealed that the majority of conserved aa were located after the leucine residue corresponding to aa 87 of *S. agalactiae* and aa 90 of *S. uberis* CAMP. Approximately the first third of the two CAMP proteins encode non-identical signal sequences of 29 aa (*S. agalactiae*) and 30 aa (*S. uberis*), followed by regions of 57 aa and 59 aa respectively which do not share extensive homology.

Initial attempts to purify the expressed 6 × His-tagged (6 × His)CAMP-3 fusion protein from pET-CAMP-3 by metal chelate affinity chromatography were unsuccessful. Surprisingly, the protein did not bind the Ni-NTA column, and subsequent DNA sequencing revealed a small deletion in pET-CAMP-3, which corresponded to the 6 × His region of pET-15b. Regardless of this fact, the *camp-3* region remained intact, and it was, therefore, decided to purify CAMP-3 by anion exchange chromatography. Most of the CAMP-3 protein was found in the wash (unbound) fraction, and was concentrated for use in the final vaccine formulation.

3.3. Challenge of vaccinated animals with S. uberis

Serum and milk anti-(6 × His)GapC and anti-CAMP-3 IgG and IgA titres were determined by ELISA. Table 2

Table 2
Anti-GapC and anti-CAMP IgG and IgA titres[a]

Antigen		Group	IgG titres		IgA titres	
			Serum	Milk	Serum	Milk
GapC	Pre-challenge	1	8.33 (±0.87)	4.28 (±0.55)	2.75 (±0.85)	0.86 (±1.10)
		2	12.74 (±1.64)	7.12 (±0.34)	3.39 (±0.31)	2.11 (±1.22)
		3	13.21 (±0.84)	7.85 (±1.09)	2.72 (±1.50)	1.24 (±1.33)
		4	9.41 (±0.69)	4.51 (±0.61)	2.09 (±1.03)	1.34 (±1.26)
	Post-challenge	1	2.42 (±3.75)	6.38 (±0.55)	4.20 (±0.97)	1.35 (±1.55)
		2	10.12 (±1.34)	6.66 (±0.22)	4.78 (±0.94)	1.14 (±1.25)
		3	10.79 (±1.07)	9.05 (±1.64)	4.64 (±1.02)	1.31 (±1.22)
		4	5.30 (±4.14)	5.84 (±0.46)	3.49 (±3.28)	1.80 (±1.25)
CAMP	Pre-challenge	1	7.35 (±1.00)	4.06 (±0.39)	1.63 (±1.53)	0.60 (±0.81)
		2	7.98 (±0.98)	5.33 (±0.34)	0.90 (±1.36)	1.77 (±0.90)
		3	7.21 (±0.99)	5.06 (±0.70)	2.56 (±1.90)	1.67 (±1.42)
		4	11.82 (±0.59)	8.37 (±1.02)	3.69 (±2.31)	1.46 (±1.44)
	Post-challenge	1	6.19 (±4.82)	4.87 (±1.37)	0	0.58 (±0.94)
		2	6.98 (±3.52)	5.07 (±0.78)	1.46 (±2.42)	0.41 (±0.99)
		3	6.41 (±5.24)	5.26 (±1.00)	2.66 (±2.24)	1.85 (±1.42)
			13.47 (±0.55)	8.90 (±1.13)	5.09 (±1.42)	1.90 (±1.30)

[a] Groups shown are (1) control, (2) *S. dysgalactiae* (6 × His)GapC, (3) *S. uberis* (6 × His)GapC, and (4) CAMP-3 vaccinates. Pre-challenge data correspond to serum IgG titres at day 28, and serum IgA, milk IgG and IgA titres at day 21. Post-challenge data correspond to serum IgG titres at day 47, and serum IgA, milk IgG and IgA titres at day 43.

shows pre- and post-challenge titres, presented as the arithmetic means of the natural log transformed values of serum titres from all animals in each treatment group (standard deviations in parentheses).

Prior to vaccination, only four animals showed any detectable serum IgG titre against (6 × His)GapC. Following vaccination, all animals vaccinated with (6 × His)GapC showed a significant increase in both serum and milk anti-(6 × His)GapC IgG titres, which consistently remained at least 10-fold higher than the control animals, while anti-(6 × His)GapC IgG titres in animals vaccinated with CAMP-3 were similar to those of the control group. Anti-(6 × His)GapC IgG titres in milk were consistently lower than the corresponding values in serum. However, immediately prior to challenge the increased serum and milk IgG titres in (6 × His)GapC vaccinated animals, compared to control and CAMP-3 vaccinated animals, was apparent. Serum anti-(6 × His)GapC IgA levels were detectable in all groups prior to challenge, but rose significantly following challenge. Even the CAMP-3 vaccinated group showed an increase in serum anti-(6 × His)GapC IgA titres, most likely resulting from exposure to the cell surface-associated GapC of the *S. uberis* challenge bacteria. In CAMP-3 vaccinated animals, a post-challenge increase in anti-(6 × His)GapC milk IgG titres was also observed, although a corresponding increase was not observed in serum IgG titres. In contrast to serum, milk anti-(6 × His)GapC IgA was virtually undetectable in all groups, both pre- and post-challenge.

Following vaccination of cows with CAMP-3, there was a marked increase in serum and milk anti-CAMP IgG titres, compared to those of the control and (6 × His)GapC vaccinated animals. Furthermore, in contrast to anti-(6 × His)GapC IgG titres, anti-CAMP-3 titres increased post-challenge, whereas those for (6 × His)GapC decreased slightly. Although the cause is unknown, this observation was consistent throughout all vaccine groups; post-challenge serum anti-(6 × His)GapC IgG titres were found to have decreased, whereas the corresponding titres in milk had increased (with the exception of the *S. dysgalactiae* (6 × His)GapC vaccinated group). Pre-challenge, serum anti-CAMP-3 IgA titres were higher than the equivalent serum anti-(6 × His)GapC titres determined at the same time point (day 21), and at the time the post-challenge serum samples were taken, anti-CAMP-3 IgA levels had increased in all groups, most significantly in those vaccinated with CAMP-3. In contrast, both pre- and post-challenge milk anti-CAMP-3 IgA titres were virtually undetectable.

Following challenge with *S. uberis* SU21, at no point were bacteria recovered from any animals, vaccinated or otherwise. This is consistent with the results of a previous study [20], where no bacteria were isolated following challenge from dairy cows vaccinated with heat-killed *S. uberis*, although bacteria were isolated from the unvaccinated control animals. It is possible that in the current study the inoculum administered was low enough to induce mastitis without causing persistent infection, even in unvaccinated animals. However, despite the absence of recoverable bacteria, animals did display clinical signs of disease, and SCC indicated that inflammation had occurred. Therefore, the challenge was deemed successful. Between vaccine groups, no significant differences were observed in rectal temperatures, and clinical scores determined that there were no significant differences in the severity of infection (data not shown). Although no differences in milk yield were observed in any animals, the quality of milk was slightly affected in all groups, as discussed below.

The bovine udder is comprised of unconnected quarters, and by challenging only two quarters with *S. uberis* an internal control was provided for each animal. Fig. 1 shows SCC over the course of the trial, for each particular vaccine group. On initial analysis, the reported SCC of the control group appear somewhat variable; however, the overall relationship is an increase of SCC over time, explained by a quadratic relationship ($P = 0.02$). After day 2, SCC of the control group increased markedly, reaching their highest level at day 4 post-challenge. SCC then decreased slightly, before rising markedly again by day 7 post-challenge. This somewhat erratic trend is consistent with SCC values reported elsewhere, following challenge of lactating cows with *S. uberis* [19,20]. The SCC of *S. dysgalactiae* (6 × His)GapC vaccinated animals increased sharply immediately post-challenge, reaching a maximum at day 3, before decreasing erratically over the remainder of the trial. Nevertheless, at no point was the decrease in SCC statistically significantly different from that of the control group, despite an apparent difference from day 4 post-challenge onward. Vaccination with *S. uberis* (6 × His)GapC resulted in a significant decrease in SCC, compared to the control group. From day 3 onward, SCC in this group were statistically significantly lower than those of the control group (*P*-values of 0.023 at day 3, 0.001 at day 4, 0.011 at day 5, 0.006 at day 6, and 0.000 at day 7 post-challenge). SCC of cows vaccinated with the CAMP-3 antigen were slightly higher than those of the *S. uberis* (6 × His)GapC vaccinated animals, although they were still clearly lower than those of the control group. Comparison of SCC of the control and CAMP-3 vaccinated groups revealed statistically significant differences at days 3 (*P*-value of 0.033), 6 and 7 post-challenge (*P*-values of 0.032, and 0.046, respectively), but not at days 4 and 5, even though SCC were obviously lower in the CAMP-3 vaccinated group on these days.

Following challenge with SU21, the time that milk quality remained affected varied between vaccine groups. Post-challenge, milk quality in the control, *S. dysgalactiae* (6 × His)GapC, CAMP-3, and *S. uberis* (6 × His)GapC vaccinated groups was reduced for a total of 21, 24, 11, and 9 days respectively. According to this data, mastitis in the *S. dysgalactiae* (6 × His)GapC vaccinated group was no less severe, if not worse, than that of the control group. Conversely, although vaccination with *S. uberis* (6 × His)GapC did not completely prevent reduced milk quality, it did

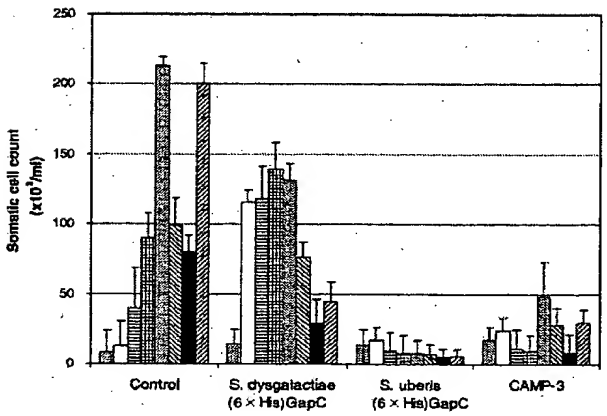

Fig. 1. Geometric mean SCC (plus 1 standard deviation) in quarters challenged with *S. uberis* were determined for each vaccine group, for 7 days following challenge. Data sets correspond to day 0 (▨), day 1 (□), day 2 (▤), day 3 (▦), day 4 (▩), day 5 (▧), day 6 (■), and day 7 (▨). Despite the appearance of the figure, the SCC for *S. dysgalactiae* (6 × His)GapC vaccinated animals on days 7 and 8, and CAMP-3 vaccinated animals on days 4 and 5 post-challenge are not statistically significantly different from those of the control.

significantly reduce the length of time that milk quality was affected. Vaccination with CAMP-3 also appeared to reduce the length of time that milk quality was reduced, although not as much as in the *S. uberis* (6 × His)GapC group, which is in keeping with the SCC results.

4. Discussion

With a view to identifying cross-protective protein antigens for use as potential vaccines against mastitis-causing streptococci, we have found that in addition to *S. dysgalactiae*, the pathogenic streptococci *S. uberis* and *S. agalactiae* also possess cell wall-associated GapC (data not shown). The *S. dysgalactiae* (6 × His)GapC was included in this study to determine whether its previously observed protective capacity against *S. dysgalactiae* infection (unpublished data), would also result in cross-species protection against infection by *S. uberis*, due to the ca. 92% homology observed between the two proteins. Most animal vaccines employ killed bacterial cells, or crude mixtures of proteins to elicit protection, however, in this study we have used defined sub-unit vaccines to elicit greater protection than has been observed in previous trials with *S. uberis* [19–21].

The possibility that sequences at the 5′- and 3′-ends of the *gapC* genes described in this study were altered cannot be discounted, due to the fact that the PCR primers used for *gapC* amplification, based on the sequences of the *gapC* genes of *S. dysgalactiae* and *S. equisimilis*, and the *plr* gene of *S. pyogenes*, were intragenic. During the preparation of this manuscript, the sequence of the gene encoding the *S. agalactiae* surface antigen delta was submitted to GenBank [24] under the accession number AF338416. This antigen is identical to GapC, and comparison of the reported sequence with the *S. agalactiae gapC* sequence from our study revealed 100% homology over the region of our 5′-PCR primer. In the region of the 3′-PCR primer, two mismatched bases were present; at bp 993, a tryptophan residue in our sequence was reported as a cytosine, and at bp 1002 a cytosine residue in our sequence was reported as a tryptophan. However, both of the mismatched bases between the two *S. agalactiae* sequences correspond to the tertiary nucleotide in a codon, and their predicted aa sequences remain identical.

Vaccination with *S. dysgalactiae* (6 × His)GapC did not result in protection against mastitis. This may be because the (6 × His)GapC of *S. dysgalactiae* is not as protective as that of *S. uberis*, despite sharing ca. 92% similarity. Preliminary PileUp analysis of the predicted aa sequences of GapC proteins described in this study, with the GapC/Plr proteins of *S. dysgalactiae*, *S. equisimilis*, and *S. pyogenes*, revealed ca. five distinct non-conserved regions (data not shown), and it is possible that these regions correspond to epitopes important in eliciting protection.

Previous work [20] has shown that vaccination of dairy cows against mastitis pathogens can result in a rapid and intense inflammatory response, indicated by large numbers of SCC in milk, following bacterial challenge. This hypersensitivity, resulting from increased neutrophil recruitment into the mammary gland following infection, is undesirable, since it is the inflammation itself that defines the mastitic

condition, and the elevated somatic cell levels are the major contributing factor to economic losses to the dairy industry through discarded milk. Vaccination with *S. uberis* (6 × His)GapC resulted in significant protection against heterologous challenge, concluded from the fact that milk SCC did not increase significantly, whereas those of the challenged, unvaccinated animals did. This suggests that *S. uberis* (6 × His)GapC is ideally suited as a vaccine against *S. uberis* mastitis, and it is likely that protection extends against a wider range of heterologous strains than the one used in this study.

It is not yet known why vaccination with *S. uberis* (6 × His)GapC prevented inflammation of the mammary gland following challenge. Interestingly, mammalian GAPDH has been shown to be involved in a number of cellular processes in addition to its role in glycolysis, including membrane fusion, microtubule bundling, phosphotransferase activity, nuclear RNA export, DNA replication, and DNA repair. Further studies have also shown GAPDH to be involved in apoptosis, prostate cancer, viral pathogenesis, and neurodegenerative disease (for review, see [25]). Given its multifunctional role in mammalian physiology, it is intriguing to think that it might also be involved in the inflammatory process. Therefore, possible cross-reactivity between mammalian GAPDH and anti-(6 × His)GapC antibodies potentially resulted in a decrease in circulating GAPDH, and hence a decrease in inflammation. Alternatively, a previous study, using a live *S. uberis* vaccine, also reported protection against challenge, without the associated influx of PMN's into the mammary gland [20]. The protective effect of the vaccine was thought to decrease the rate of gland colonization by *S. uberis*, thus controlling infection by dilution and subsequent elimination of bacteria within the milk, prior to initiation of PMN influx. It is entirely possible that this is also true of the *S. uberis* (6 × His)GapC vaccine. Another explanation may be that in *S. uberis* (6 × His)GapC vaccinated cows, circulating anti-(6 × His)GapC antibody may inhibit cell surface-associated GapC on the *S. uberis* challenge bacteria, resulting in a decrease in bacterial virulence. This hypothesis derives from a study in which the cell surface-associated GAPDH of the eukaryotic parasite *Schistosoma mansoni* was shown to be a selective target for antibodies in the sera of humans resistant to reinfection [26]. Vaccination of mice and rats with a 22 aa B cell epitopic region of *S. mansoni* GAPDH resulted in partial protection against challenge with *S. mansoni* [27]. Subsequently, determinants of *S. mansoni* GAPDH were proposed as candidates for an anti-schistosome submit vaccine. Antibodies from sera of schistosome-infected patients inhibited the activity of recombinant schistosome GAPDH [28], and drugs inhibiting GAPDH activity also decreased parasite survival [29,30].

Vaccination with CAMP-3 also resulted in a statistically significant decrease in SCC compared to the control group, following challenge. Combined with the observation that milk quality was not affected for as long a time following challenge as the control group, it is encouraging to hypothesise that alternative vaccine formulations may increase protection. The ca. 60 % purity of the CAMP-3 protein used in this study arose through initial problems with purification of the 6 × His-tagged protein, due to a small deletion in pPolyCAMP-3, which effectively removed the histidine tail without abolishing protein expression. Subsequently, anion exchange chromatography was used to partially purify CAMP-3 from a whole cell lysate, instead of the intended metal chelate affinity chromatography. However, despite not being as pure as the (6 × His)GapC proteins, the CAMP-3 preparation was considered to be sufficiently pure for vaccination purposes. A chimeric CAMP antigen was designed for use as a single subunit vaccine antigen against *S. uberis* and *S. agalactiae* mastitis, and its protective ability against disease by the latter organism is currently under examination, along with the (6 × His)GapC proteins of *S. agalactiae* and *S. uberis*. The use of a common antigen to protect against infections by both organisms may be advantageous to both the dairy producer and the vaccine manufacturer, as incurred costs would be reduced on both sides.

Acknowledgements

For technical assistance, we thank Tracy Prysliak, and Nelson Dinn and the staff of the Dairy Education and Research Centre.

This work was supported by the Natural Sciences and Engineering Research Council of Canada, the Canadian Bacterial Diseases Network, the Saskatchewan Agriculture Development Fund, the Dairy Farmers of Canada, the Alberta Agricultural Research Institute, and the British Columbia Investment Agriculture Foundation.

References

[1] Kossaibati MA, Esslemont RJ. The costs of production diseases in dairy herds in England. Vet J 1997;154:41–51.

[2] Bramley AJ. Sources of *Streptococcus uberis* in the dairy herd. I. Isolation from bovine faeces and from straw bedding of cattle. J Dairy Res 1982;49:369–73.

[3] Kruze J, Bramley AJ. Sources of *Streptococcus uberis* in the dairy herd. II. Evidence of colonization of the bovine intestine by *Streptococcus uberis*. J Dairy Res 1982;49:375–9.

[4] Hogan JS, Smith KL, Hoblet KH, et al. Field survey of clinical mastitis in low somatic cell count herds. J Dairy Sci 1989;72:1547–56.

[5] Anderson JC. The problem of immunisation against staphylococcal mastitis. Br Vet J 1978;134:412–20.

[6] Almeida RA, Oliver SP. Antiphagocytic effect of the capsule of *Streptococcus uberis*. Zentralbl Veterinarmed [B] 1993;40:707–14.

[7] Oliver SP, Almeida RA, Calvinho LF. Virulence factors of *Streptococcus uberis* isolated from cows with mastitis. Zentralbl Veterinarmed [B] 1998;45:461–71.

[8] Schaufuss P, Sting R, Schaeg W, Blobel H. Isolation and characterisation of hyaluronidase from *Streptococcus uberis*. Zentralbl Bakteriol 1989;271:46–53.

[9] Leigh JA. Purification of a plasminogen activator from *Streptococcus uberis*. FEMS Microbiol Lett 1994;118:153–8.

[10] Rosey EL, Lincoln RA, Ward PN, Yancey Jr RJ, Leigh JA, Pau A. A novel plasminogen activator from *Streptococcus uberis*. FEMS Microbiol Lett 1999;178:27–33.
[11] Fang W, Oliver SP. Identification of lactoferrin-binding proteins in bovine mastitis-causing *Streptococcus uberis*. FEMS Microbiol Lett 1999;176:91–6.
[12] Lopes MF, Merquior VL, Peralta JM, Teixeira LM. Partial characterization of the cohemolytic factor produced by *Streptococcus uberis* and comparison with the CAMP-factor. FEMS Immunol Med Microbiol 1995;12:205–12.
[13] Jiang M, Babiuk LA, Potter AA. Cloning, sequencing and expression of the CAMP factor gene of *Streptococcus uberis*. Microb Pathog 1996;20:297–307.
[14] Jurgens D, Sterzik B, Fehrenbach FJ. Unspecific binding of group B streptococcal cocytolysin (CAMP-factor) to immunoglobulins and its possible role in pathogenicity. J Exp Med 1987;165:720–32.
[15] Lottenberg R, Broder CC, Boyle MD, Kain SJ, Schroeder BL, Curtiss Rd. Cloning, sequence analysis, and expression in *E. coli* of a streptococcal plasma receptor. J Bacteriol 1992;174:5204–10.
[16] Pancholi V, Fischetti VA. A major surface protein on group A streptococci is a glyceraldehyde-3-phosphate-dehydrogenase with multiple binding activity. J Exp Med 1992;176:415–26.
[17] Pancholi V, Fischetti VA. Glyceraldehyde-3-phosphate dehydrogenase on the surface of group A streptococci is also an ADP-ribosylating enzyme. Proc Natl Acad Sci USA 1993;90:8154–8.
[18] Hill AW, Finch JM, Field TR, Leigh JA. Immune modification of the pathogenesis of *Streptococcus uberis* mastitis in the dairy cow. FEMS Immunol Med Microbiol 1994;8:109–17.
[19] Finch JM, Winter A, Walton AW, Leigh JA. Further studies on the efficacy of a live vaccine against mastitis caused by *Streptococcus uberis*. Vaccine 1997;15:1138–43.
[20] Finch JM, Hill AW, Field TR, Leigh JA. Local vaccination with killed *Streptococcus uberis* protects the bovine mammary gland against experimental intramammary challenge with the homologous strain. Infect Immun 1994;62:3599–603.
[21] Leigh JA, Finch JM, Field TR, et al. Vaccination with the plasminogen activator from *Streptococcus uberis* induces an inhibitory response and protects against experimental infection in the dairy cow. Vaccine 1999;17:851–7.
[22] Podbielski A, Blankenstein O, Lutticken R. Molecular characterization of the *cfb* gene encoding group B streptococcal CAMP-factor. Med Microbiol Immunol (Berl) 1994;183:239–56.
[23] van Drunen Littel-van den Hurk S, Parker MD, Massie B, et al. Protection of cattle from BHV-1 infection by immunization with recombinant glycoprotein gIV. Vaccine 1993;11:25–35.
[24] Seifert KN, Bleiweis AS, McArthur WP, Brady LJ. The group B streptococcal surface antigen delta is a glyceraldehyde-3-phosphate dehydrogenase. GenBank submission, 2001.
[25] Sirover MA. New insights into an old protein: the functional diversity of mammalian glyceraldehyde-3-phosphate dehydrogenase. Biochim Biophys Acta 1999;1432:159–84.
[26] Dessein AJ, Begley M, Demeure C. Human resistance to *Schistosoma mansoni* is associated with IgG reactivity to a 37-kDa larval surface antigen. J Immunol 1988;140:2727–36.
[27] Argiro LL, Kohlstadt SS, Henri SS. Identification of a candidate vaccine peptide on the 37 kDa *Schistosoma mansoni* GAPDH. Vaccine 2000;18:2039–48.
[28] Argiro L, Dorrig C, Lisbeuf S, Bourgois A, Romeuc JL. Production of Sm37-GAPDH, a major therapeutical target in human schistosomiasis. Biotechnol Bioeng 2000;68:136–41.
[29] Callens M, Hannaert V. The rational design of trypanocidal drugs: selective inhibition of the glyceraldehyde-3-phosphate dehydrogenase in *Trypanosomatidae*. Ann Trop Med Parasitol 1995;89(Suppl 1):23–30.
[30] Bourguignon SC, Alves CR, Giovanni-De-Simone S. Detrimental effect of nitric oxide on *Trypanosoma cruzi* and *Leishmania major* like cells. Acta Trop 1997;66:109–18.

ELSEVIER

Vaccine 20 (2002) 3047–3048

Vaccine

www.elsevier.com/locate/vaccine

Letter to the Editor

Immunisation of dairy cattle with recombinant *Streptococcus uberis* GapC or a chimeric CAMP antigen confers protection against heterologous bacterial challenge M.C. Fontaine et al. [Vaccine 20 (2002) 2278–2286]

The above article, published in a recent volume of *Vaccine* is misleading, in that it claims that protection from mastitis was achieved by vaccination when this cannot be substantiated from the observations.

1. The challenge dose was 3 ml of a bacterial suspension in saline containing 3.0×10^7 cfu/ml, a total of 9.0×10^7 cfu. This dose exceeds (by a factor of 10^5) that used typically by others in similar challenge models [1–8], in which intramammary infection and mastitis were induced. However, use of such a high dose is not in itself a problem or a cause for concern if protection from challenge is to be established.
2. Following challenge bacteria were not recovered from any mammary quarter of vaccinated or non-vaccinated animals. Since a typical mammary quarter can produce around 2–4 l of milk at any one milking, it is likely that (in the absence of bacterial killing and/or bacterial growth) the number of bacteria recovered in milk should have been in the order of 10^4 cfu/ml. Such numbers would be detected easily by routine and simple bacteriological techniques. Even allowing for no bacterial growth and killing of 99% of the challenge inoculum, around 10^2 cfu/ml should have been present in the milk. This number is also detectable by routine and simple bacteriology. The possibility that bacteria were present in reasonable numbers in vivo but were not detected following analysis of the milk samples can be discounted, as none of the animals exhibited inflammatory cell responses consistent with such a scenario [1–8]. Since bacteria were not detected, even transiently, within the control group of animals, it can only be concluded that either the inoculum was not viable or that the organism was totally avirulent or that all the animals, including the non-vaccinated controls, were resistant to infection.

   Given the incidence of *Streptococcus uberis* infection and mastitis in dairy cattle the latter is highly unlikely.

   The virulence of the organism could be questioned, as studies in our laboratory have identified single genes that are *essential* for infection within the bovine mammary gland (Leigh et al., unpublished). Challenge with strains lacking such genes (even with 10^5 cfu) does not result in infection or disease. Bacteria, however, can still be detected in the milk for up to 24 h following initial exposure (Field et al., unpublished).

   The most likely explanation of the data presented in this paper is that the inoculum had lost viability prior to infusion within the mammary gland. Loss of viability of streptococci suspended in saline has been observed occasionally in our laboratory for both *S. uberis* and *S. suis*. In order to check numbers of bacteria present in challenge doses it is necessary to estimate viable numbers both prior to and following administration of the challenge.
3. The claim that the data in this communication was in any way consistent with that generated from our laboratory in which heat or formalin killed bacterial cells [5], live bacterial cells [6,7] or the plasminogen activator, PauA [8], were used as vaccine antigens is unfounded. In all of our studies the non-vaccinated, control animals became infected (and shed bacteria in the range of 10^6–10^8 cfu/ml) and exhibited clinical signs of disease (somatic cell counts of around 10^7 cells/ml). These events were not detected in the control animals in this communication. Bacteria could not be detected and the major clinical response to challenge was the presence of a somatic cell count of around 200,000–225,000 cells/ml on only 2 days (days 4 and 7) of the 7 days monitored, post-challenge. Cell counts of this order are not uncommon even in milk obtained from uninfected mammary quarters, and all of the remaining samples contained less than 150,000 cells/ml, which is typical for milk from the non-infected, lactating, bovine mammary gland.

This communication provides no evidence that the challenge used established intramammary infection or induced mastitis in any animal, including the non-vaccinated control group. In conclusion, this communication describes a study in which the control group did not perform as predicted

PII: S0264-410X(02)00263-3

or required. In short, it is a report of an uncontrolled experiment in which the protective effect of GapC cannot be evaluated.

References

[1] Paape MJ, Schultze WD, Corlett NJ, Wienland BT. Effect of abraded intramammary device on outcome in lactating cows after challenge exposure with *Streptococcus uberis*. Am J Vet Res 1988;49:790–2.

[2] Nickerson SC, Boddie RL, Owens WE, Watts JL. Effects of novel intramammary devices models on incidence of mastitis after experimental challenge. J Dairy Sci 1990;73:2774–84.

[3] Smits E, Burvenich C, Guidry AJ, Roets E. In vitro expression of adhesion receptors and diapedesis by polymorphonuclear neutrophils during experimentally induced *Streptococcus uberis* mastitis. Infect Immun 1998;66:2529–34.

[4] Hockett ME, Hopkins FM, Lewis MJ, Saxton AM, Dowlen HH, Oliver SP, et al. Endocrine profiles of dairy cows following experimental induced mastitis during early lactation. Anim Reprod Sci 2000;58:241–5.

[5] Finch JM, Hill AW, Field TR, Leigh JA. Local vaccination with killed *Streptococcus uberis* protects the bovine mammary gland from experimental infection following intramammary challenge with the same strain. Infect Immun 1994;62:3599–603.

[6] Hill AW, Finch JM, Leigh JA, Field TR. Immune modification of the pathogenesis of *Streptococcus uberis* mastitis in the dairy cow. FEMS Immunol Med Microbiol 1994;8:109–18.

[7] Finch JM, Winter A, Walton A, Leigh JA. Further studies on the efficacy of a live vaccine against mastitis caused by *Streptococcus uberis*. Vaccine 1997;15:1138–43.

[8] Leigh JA, Finch JM, Field TR, Real NC, Winter A, Walton AW, Hodgkinson SM. Vaccination with the plasminogen activator from *Streptococcus uberis* induces an inhibitory response and protects against experimental infection in the dairy cow. Vaccine 1999;17:851–7.

J.A. Leigh
Institute for Animal Health, Compton Laboratory
Compton, Newbury, Berkshire RG20 7NN, UK
Tel.: +44-1635-577249; fax: +44-1635-577243
E-mail address: james.leigh@bbsrc.ac.uk (J.A. Leigh)